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METHOD FOR THE LIGHT-INDUCED IMMOBILIZATION OF BIOMOLECULES ON CHEMICALLY "INERT" SURFACES

[Verfahren zur Lichtinduzierten Immobilisierung von Biomolekülen an chemisch "inerten" Oberflächen]

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DESCRIPTION OF INVENTION

This invention relates to a process for the photo-induced immobilization of biomolecules in monomolecular layers using photoactivable arylazides or diazirines as molecular adhesives.

Triggered by the rapid development and miniaturization of bioanalytical methods, on the one hand, and progress made in biosensor technique, on the other hand, there is great interest in developing a better understanding of the reciprocal interaction between biomolecules and surfaces of organic-synthetic or inorganic nature. Of equal importance is the development of methods which produce effective, chemically stable coupling of biomolecules on carrier materials whereby the former must be neither chemically pretreated nor exposed to extreme (coupling) reaction conditions. Analytic/diagnostic methods and reproduction of surface-active biosensors demand the suitable anchoring of the active substances in monomolecular layers. Because the surface of many carrier materials used for this purpose so far do not have any or only little suitable chemical reactive functions, the biomolecules so far have been bound mostly in a surface-covering manner to pretreated carrier materials by means of specifically group-related modification reactions. Chemical immobilization of this kind are basically possible. But the method presupposes the presence of functional

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[1]Numbers in the margin indicate pagination in the foreign text.

groups that can be activated in a specifically targeted manner. In addition, the monomolecular occupation of surfaces with hitherto used methods can be performed merely in a surface-covering manner (bulk process).

The object of the invention is to bind macromolecular substances, in particular, biomolecules to chemically "inert" surfaces that will be oriented with respect to a specific region and also topologically. The intent is to point up methods that will make it possible to immobilize monomolecular layers of biologically-active active substances (albumin molecules, nucleic acids, carbohydrates, lipids, low-molecular active substances) by suitable crosslinking agents upon solid phases (carrier material) of differing chemical nature. Photoactivable crosslinking agent molecules are to be used for covalent immobilization. Photoactivable reagents are superior to the thermochemical crosslinkage reactions because the reaction can be selected photooptically or by means of specifically targeted application of electrical energy regarding place and dimension and because the coupling reaction can be triggered in a time-controlled manner.

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This work describes two processes whose use leads to the photochemical immobilization of biomolecules on "inert" carriers. The covalent bonding of the biomolecules upon the carrier takes place via photogenerated carbines or nitrenes. Carbenes, just as

nitrenes, are chemically extremely reactive intermediate products. They are suitable for binding biomolecules in a covalent manner by means of insert reactions in C-H, C-C, C=C, N-H, O-H, S-H bonds. The resulting broad reaction spectrum of the photogenerated carbenes and nitrenes therefore exceeds the thermochemical modification reactions as regards the required reaction conditions. By employing laser light sources or by applying the energy required for the activation of the reactive functions, one can selectively activate the smallest surfaces and one can occupy them with biomolecules in a monomolecular fashion.

The photochemically induced immobilization of biomolecules can be accomplished in a single reaction step using a multiple derived linker molecule. On the other hand, one can also implement a two-stage process. The latter presupposes the covalent chemical coupling of a (low-molecular) linker molecule upon the carrier.

The first-named, single-phase coupling method consists of the following substeps.

1. A linker molecule (for example, a synthetic or natural polymer) is derived several times (= photolinker polymer) with heterobifunctional, photoactivable crosslinker molecules (for example, 3-(trifluoromethyl)-3-(m-isothiocyanophenyl)diazirine or 3-(trifluoromethyl)-3-(m-aminophenyl)diazirine.
2. The photolinker polymer is dried upon the "inert" surface.

3. Biomolecules that are to be immobilized are applied upon the photolinker-occupied surface. The solvent is removed partially or entirely.

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4. The photoactivable functional groups are activated and the coupling reaction is triggered by irradiating light with a suitable wavelength (diazirine: 350 nm).
5. After photocoupling, non-bond biomolecules are removed by repeatedly washing the surface (for example, by means of filtration). With the help of this step, one can simultaneously exchange accompanying substances (buffer components, salts, detergents) or remove them out of the system.

The following substeps are necessary for the two-phase immobilization process:

1. The carrier material (for example, glass, polymeric materials, silicon oxide, mica) is occupied with functional groups according to known methods (for example, introduction of primary or secondary amines, carboxyl groups, thiol functions).
2. Carrier material, thus modified, is derived with heterobifunctional, photoactivable crosslinker molecules (for example, p-azidophenyl-isothiocyanate, 3-(trifluoromethyl)-3-(m-isothiocyanophenyl)diazirine, p-azidoaniline, 3-(trifluoromethyl)-3-(m-amino-

phenyl)diazirine, or N-(4-azidophenylthio)-phthalimide) derivatives and are mixed in with the photoactivable carrier.

3. For immobilization, the biomolecules are placed in contact with the photoactivable carrier material by dipping into solutions, by dripping on (adsorption) or by means of electrophoretic methods (electroblotting).

4. The carriers are activated under inert gas by irradiating light of defined wavelength or application of corresponding energy (arylazides, 260 nm; diazirines, 350 nm). The coupling reaction is triggered in this way.

5. After completed coupling, the non-bound biomolecules are removed by repeated washing of the carrier or by filtration. During this step, one can simultaneously exchange or remove accompanying substances (buffer components, salts, detergents).

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6. This means that the carriers, occupied by biomolecules, are now ready for use. If the carrier material is to be covered by means of regionally selective activation of the surface in a specifically regionally related manner with several biomolecules (for example, receptors, enzymes, immunoreagents), then steps 3 to 6 can be repeated several times. Possible practical applications are described in Claims 2 to 6.

Example of Using the Single-Phase Coupling Process

In analogy to the existing immunological methods, one can immobilize proteins (Streptavidine, immunoglobulins) and, for the first time, also nucleic acids in an astonishingly simple process upon microtiter plates in a covalent manner. The process (PhotoLink) does not demand any special pretreatment of the biomolecule to be bonded and the commercially available carrier materials (for example, Nunc Immunoplate Maxisorp) can be used without any pretreatment. Microtiter plates are covered with a polymer (polypeptide) that previously has been equipped several times with photoactivable functional groups (photolinker peptide). Immobilization takes place after exposure by means of carbene insertion. The photoactive functional groups (for example, diazirines) that have been applied to the carrier molecule react simultaneously with the molecule that is to be bonded (for example, protein, DNS, immunoglobulin) and with the surface to be covered (for example, polystyrene).

Production of the Photolinker Peptide

Bovine serum albumin (80 mg) is suspended in 14 ml TEA/HAc buffer, pH 10.5 (100 ml H_2O, 100 ml acetone, 1 ml triethylamine, 1 ml acetic acid (2M)) and is exposed to ultrasonic waves in the ultrasound bath until the solution is clear. One adds 6 ml of acetone to 24 µl of 3-(trifluoromethyl)-3-(m-aminophenyl) diazirine (TRIMID, made according to Dolder et al., (1990) J. Prot. Chem. 9, 407-415) in carbon tetrachloride. The protein

solution and the reagent are mixed in a 100 ml round flask and are refluxed for one hour at 70°C. The reaction solution is then extracted three times with 30 ml each of heptane/acetic acid ethylester (6:3 v/v) and the organic phase is discharged. The water phase is lyophilized overnight. The dry product is suspended in 6 ml of 0.4% (w/v) of sodium dodecyl sulfate in PBS

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(150 mM NaCl, 5 mM of sodium phosphate, pH 7.4) and is exposed to ultrasound until the solution is clear. For further purification, the product is chromatographed on Sephadex G-15 medium in PBS and the combined protein-containing fractions are dialyzed for 48 hours against H_2O bidest (Spectrapor cut off 6000-8000). After lyophilization, the product is kept at -20°C.

Occupying the "Inert" Surface

The reaction vessels of the titer plates (Nunc-Immuno Module, Polysorp F8) are mixed with 40 µl each of photolinker peptide in H_2O (corresponding to 1 nMol TRIMID derived bovine serum albumin). The bottom of the reaction vessel should be wetted uniformly. The reaction vessels are then dried in the water jet vacuum for one hour at room temperature. Titer plates thus occupied can, when packaged in a light-proof manner, be kept at -20°C for at least 3 months.

Application of Biomolecules and Light-Induced Immobilization

The biomolecules (ligands), used for immobilization, are dissolved in any buffer system (for example, 1 mg of Streptavidine in 2 ml PBS) and are diluted up to the desired final concentration (for example, 10 to 1,000 pMol Streptavidine per 30 µl). The reaction vessels, covered with photolinker peptide, are mixed with 30 ml of ligand solution and are dried in the water jet vacuum for 2 hours at room temperature. For photoactivation, the reaction vessels are exposed for 5-30 minutes of radiation with UV light sources (for example, parallel-arranged UV (366 nm) tubes, Silvania F8T5/BLB USA, 8 watts or mercury vapor lamp HBO 350, Osram with the filter combination described in page 11 [sic] and are then washed 5 times each with PBS, 5 times with H_2O and twice with alcohol.

Quantitative Evidence of Immobilization

The immobilization of Streptavidine is quantified by adding radioactively marked [^{14}C]-biotin. Immunoglobulins that were immobilized according to the described method can be complexed with a second antibody that carries phosphatase in a covalent bound manner and can thus convert the substrate p-nitrophenylphosphate. Released p-nitrophenol is determined quantitatively by means of absorption measurement (405 nm) in

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commercially available ELISA-Reader units. In an analogous procedure, DNS, marked with Digoxigenine, can be coupled and can

be documented by means of anti-Digoxigenine antibodies and alkaline phosphatase.

The covalent immobilization of biomolecules of various classes illustrates the broad practical application spectrum and the great practical application potential of the extensively standardized method. The yields of bound molecules are good. The method can be integrated without any restrictions into existing analysis processes (for example, ELISA). The sensitivity of evidence is within the range of analogous methods that are based on thermochemical immobilization (for example, formation of an amide bond, borhydride reduction). Along with independence from functional groups on the ligand and independence from restricting reaction conditions, the multiple uses of the antigen-occupied microtiter plates and the easily performed coupling of proteins and nucleic acids are of analytical and process engineering significance.

The method for the first time represents a usable, tested method for the immobilization of biomolecules, which could be developed all to marketing readiness. An important advantage inherent in the described procedure is represented by the fact that the diazirines can be handled at daylight that has been filtered through window glass. Their activation takes place at 350 nm with commercially available illumination equipment.

Example of a Use of Two-Step Immobilization

The process will now be described with the help of the sequence analysis of a hexapeptide. Representing biologically active peptides that occur in great dilution in body fluids, we use a soluble peptide in this process with the amino acid sequence NH_2-Leu-Trp-Met-Arg-Phe-Ala-COOH. Photocoupling of the peptide upon diazirine-derived glass filters and subsequent sequence analysis according to Edmann are described.

Production of Photoactivable Glass Fiber Filters

Glass fiber filters (for example, Firma Whatman) are activated with anhydrous trifluoroacetic acid for a period of 1 hour at room temperature and are mixed with 3-(triaethoxysilyl)-propylamine. After washing out the excess of reagent, the glass fiber membrane is treated at 50°C (1 hour). The degree of occupation with amino functions is determined analytically.

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On the average, one can detect 10 to 15 nMol amino functions per milligram of glass fiber filter. The chemical coupling of 3-(trifluoromethyl)-3-(m-isothiocyanophenyl)diazirine to aminopropylated glass is done with a 10-fold excess of crosslinker. (To make arylazide glass, one proceeds in a similar manner and mixes the aminopropylated carrier material with 4-azidophenylisothiocyanate). The reaction is performed at 40°C in cyclohexane and is ended after 90 minutes. The derived glass fiber filters are then washed with organic solvents by means of filtration.

Photocoupling the Peptides

For photocoupling purposes, the peptide 500 pmol, dissolved in 15 µl of water) is allowed to drip on the photoactivable glass fiber filter, it is rinsed all around with argon and it is then irradiated for 16 minutes with filtered light in a mercury vapor lamp (HBO 350, Osram, 200 watt initial output). The selected filter combination (WG 320 long pass filter, Firma Schott; 1 cm saturated copper sulfate solution) causes the light of the wavelength under 320 nm to be absorbed effectively. After photocoupling, the glass fiber filter, occupied with peptide, is washed with solvents of differing polarity (NaCl, 1 M; water; ethanol; chloroform; toluene).

Sequence Analysis of Peptide Coupled by Means of Photoactivation

The gas phase sequence method is suitable for sequence analysis. The coupling efficiency, measured by the yield of the N-terminal amino acid, amounts to 10%. NO sequence information can be derived from control experiments that are performed with photoactivable glass fiber filters and hexapeptide but without light activation.

CLAIMS /8

1. Process for light-induced immobilization of biomolecules (for example, albumin substances, nucleic acids, carbohydrates, lipids) for the purpose of analytical, diagnostic, medical and/or commercial use, characterized in that the biomolecule in monomolecular layers can be bound in

a covalent manner on photoactivable carrier materials by irradiation of light or application of electrical energy.

2. Process according to Claim 1, characterized in that albumin molecules, carbohydrates or nucleic acids can be bound in a covalent manner for the purpose of chemical analysis and/or structural clarification upon photoactivable carrier materials.

3. Process according to Claim 1, characterized in that immunologically active molecules (for example, antigens, antibodies, haptenes), preserving biological activity, can be bound in a covalent manner upon photoactivable carrier materials.

4. Process according to Claims 1 and 3, characterized in that biologically active molecules, in particular, enzymes, receptors, immunologically active molecules, are immobilized for the purpose of production of biosensors in a photochemical manner or by the application of electrical energy.

5. Process according to Claim 1, characterized in that albumin molecules, lipids and/or carbohydrates are bound in a covalent manner for the purpose of avoiding rejection of substances alien to the body in a photochemical manner upon surfaces of implants.

6. Process according to Claim 1, characterized in that biomolecules, in particular, albumin molecules or parts

thereof, are immobilized for the purpose of making molecular switching elements in a photochemical manner or by the application of electrical energy.